Reagentless amperometric biosensor highly sensitive to hydrogen peroxide based on the incorporation of Meldola Blue, fumed-silica and horseradish peroxidase into carbon paste

By: Haiying Liu, Zhanen Zhang, Yubo Fan, Miao Dai, Xiaolin Zhang, Jianjun Wei, Zunan Qiu, Hongbin Li, Xinxin Wu, Jiaqi Deng, Deyao Qi

H. Y. Liu, Z. N. Zhang, M. Dai, Y. B. Fan, J. Wei, Z. N. Qiu, H. B. Li, X. Wu, J. Q. Deng, and D. Y. Qi, "Reagentless Amperometric Biosensor Highly Sensitive to Hydrogen Peroxide Based on the Incorporation of Meldola Blue, Fumed Silica and Horseradish Peroxidase into Carbon Paste" *Fresenius Journal Analytical Chemistry*, 1997, 357 (3): 297-301.

Abstract:

A reagentless amperometric sensor highly sensitive to H2O2 has been prepared by incorporating fumed silica, horseradish peroxidase (HRP) and Meldola Blue into carbon paste. The efficient mediating ability to shift electrons between HRP and the carbon paste electrode via Meldola Blue was investigated by cyclic voltammetric and amperometric measurements. Reproducibility, response time, detection limit, selectivity and effects of applied potential, temperature and pH on the response of the sensor are reported. The high sensitivity of the sensor with a detection limit of 0.1 μmol/1 arose from the high efficiency of the bioelectrocatalytic reduction of hydrogen peroxide via HRP and Meldola Blue. The dependence of the Michaelis-Menten constant on the applied potential and the mediator concentration has been investigated and the results are presented.

Keywords: Methylene Blue | Fumed Silica | Anal Chim | Direct Electron Transfer | Electron Transfer Mediator

Article:

1. Introduction

Incorporation of enzymes and mediators into carbon paste matrices represents an attractive approach to the preparation of reagentless biosensors [1-5]. Carbon paste is a mixture of graphite powder and an organic liquid. The pasting liquid is immiscible with contacting aqueous solutions and includes paraffin oil, vaseline oil, nujol, silicon oil and seresin wax, which functions to fill up the gaps between the graphite particles and insulate the graphite from the contacting aqueous solution. The surface coverage of a variety of components can readily be manipulated by varying the weight of the modifier added to the paste mixture. The carbon paste-based biosensors possess many advantages including extremely low background currents, a wide operating potential window, conventional modification, renewability, miniaturization and low cost. In this paper, 7-dimethyl-amino-1,2-benzophenoxazinium salt (Meldola Blue) was used as an effective electron donor for horseradish peroxidase in a carbon paste configuration. Meldola Blue has already been employed for the electrocatalytic oxidation of reduced nicotinamide adenine dinucleotide (NADH) [6-8]. Moreover, due to its ability to mediate the oxidation of NADH, it has widely been applied to the preparation of various NAD +-dependent dehydrogenase-based biosensors for many substances such as glucose, L-lactate and ethanol [8-10]. In addition, Meldola Blue has also been utilized to shift electrons between glucose oxidase [11] or lactate oxidase [12] and the carbon paste electrode. These sensors possess a good selectivity compared with those employing other mediators such as ferrocene and tetrathiafulvalene, because the low redox potential of Meldola Blue is particularly useful for reducing the interference from electroactive species such as ascorbate and uric acid. As far as we know, the efficiency of electron transfer between horseradish peroxidase (HRP) and the carbon paste electrode via Meldola Blue has not yet been reported. In the following, the analytical performance of HRP-based carbon paste biosensors was improved by incorporating fumed silica, HRP and Meldola Blue into the paste matrix because of a prolonged retention of HRP and Meldola Blue at the silica particles. Cyclic voltammetric and amperometric measurements were employed to demonstrate the suitability of Meldola Blue as an electron transfer mediator in the bioelectrocatalytic reduction of hydrogen peroxide via HRP. The characterization of the sensor in terms of linearity, reproducibility, pH and temperature dependence, stability, selectivity and response time was investigated. The effect of the amount of Meldola Blue and the applied potential on the Michaelis-Menten constant K_m^{app} was also examined.

2. Experimental

2.1 Reagents

Meldola Blue was obtained from Aldrich, Peroxidase from horseradish (HRP) (EC 1.11.1.7, type VI) and fumed silica (particle size 7 nm, specific area 400 m 2/g) were purchased from Sigma. A solution of Eastman-AQ-55D polymer (28% dispersion) was obtained from Eastman Kodak Co., graphite powder from Fluka and paraffin oil from Merck. Hydrogen peroxide (30% wry solution) was purchased from Shanghai Chemical Reagent Company. The concentration of the dilute peroxide solutions was determined by titration with cerium (IV) to a ferroin end-point [13]. The rehydrated fumed silica was prepared according to [9]. The buffer and other chemicals were of analytical-reagent grade. 0.1 mold potassium phosphate buffer solution was used.

2.2 Preparation of the carbon paste biosensor for hydrogen peroxide.

The carbon paste biosensor was prepared by first mixing 25 mg HRP with 130 mg graphite powder, 60 mg rehydrated fumed silica and 85 mg paraffin oil, for 6 min. The desired amount of Meldola Blue was then added and mixed with the above mixture for 10 min. A platinum wire was inserted into a glass tube (2.5 mm in diameter) leaving a hole 2.0 mm deep. The resulting carbon paste was packed into the hole and the electrode surface was smoothed on a weighing paper placed over a flat glass stand. Eastman-AQ coating was cast by dipping the carbon paste electrode into Eastman-AQ (1:20 (v/v) Eastman-AQ: water) polymer solution, letting it dry in air. Other paste formulations were prepared in a similar way.

2.3 Apparatus

All experiments were performed with a three-electrode configuration comprising a carbon paste biosensor as a working electrode, a saturated calomel reference electrode and a platinum wire auxiliary electrode. The electrodes were connected to an FDH 3204 and FDH 3206 cyclic voltammetry apparatus (Scientific Equipment Company of Fudan University, China) and the signal was recorded on a type 3086 x-y recorder (Tokyo, Japan) for cyclic voltammetric and amperometric measurements, separately. All experiments were carried out in a thermostatted, stirred electrochemical cell containing 5 ml of 0.1 moll phosphate buffer (pH 7.0) at 20.0 ± 0.5°C. In the constant potential experiments, the current time response was recorded following successive additions of stock H_2O_2 solution to the buffer after a constant residual current had been established. Changes in the measured reduction current were recorded as function of time, following the addition of H_2O_2. The sensor response was measured as the difference between total and residual current.

2.4 Calculation of the Michaelis-Menten constant

The apparent Michaelis-Menten constant K_m^{app} can be determined from the electrochemical Eadie-Hofsttee form of the Michaelis-Menten equation [14]

$$j_{ss} = j_{max} - K_M^{app}(j_{ss}/C),$$

where j_{ss} the steady-state catalytic current, j_{max} stands for the maximum current measured under saturated substrate conditions, C is referred to the H_2O_2 concentration and K_m^{app} represents the apparent Michaelis-Menten constant of the system as a whole, not that of an intrinsic property of peroxidase.

3. Results and discussion

3.1 Bioelectrocatalytic reduction of hydrogen peroxide

In the absence of hydrogen peroxide, HRP gives no response. Only Meldola Blue in carbon paste produces voltammograms consistent with a quasi-reversible electron redox agent, because both the cathodic and anodic peaks of Meldola blue are proportional to the square root of the potential

scan rate ranging from 20 to 160 mV/s, and the peak-to-peak potential separation (ΔEp) is less than 190 mV. Meldola Blue displays a formal potential (E °) of - 110 mV vs SCE at pH 7.0. However, addition of hydrogen peroxide to the cell brings about a significant increase of the cathodic peak current and a decrease of the anodic peak current. Comparison of the voltammograms in the absence and the presence of hydrogen peroxide demonstrates that Meldola Blue efficiently enhances the electron communication between HRP and the carbon paste electrode in the bioelectrocatalytic reduction of hydrogen peroxide. HRP catalyzes the H_2O_2 reduction according to the following scheme [15-17]:

$$\text{HRP}(Fe^{3+}) + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (1)$$

$$\text{compound I} + \text{Med}_{red}(\text{or } e^-) \rightarrow \text{compound II} + \text{Med}_{ox} \quad (2)$$

$$\text{compound II} + \text{Med}_{red}(\text{or } e^-) \rightarrow \text{HRP}(Fe^{3+}) + \text{Med}_{ox} \quad (3)$$

Med_{red} and Med_{ox} represent the reduced and oxidized forms of the electron transfer mediator, respectively. Hydrogen peroxide oxidizes the native form of HRP in a single two-electron process, bringing about the formation of an unstable intermediate, compound I, consisting of a π-cation radical heme with Fe(IV) to which an oxygen atom is coordinated ([Fe(IV) = O]•+). In two separate one-electron transfer reactions, compound I is reduced back to its native form HRP(Fe^{3+}) through an intermediate state denoted compound II, which has heme with Fe(IV) to which OH is coordinated ([Fe(IV)OH]). In the case of HRP, the electrons necessary to close the enzymatic cycle can be donated by an electron transfer mediator or by direct electron transfer from the electrode to the heme site of the HRP in intimate contact to the conducting surface without a mediator. The electron transfer mediators employed for the HRP-based biosensors include ferrocene and its derivatives [18,19], osmium bipyridine conjugated to poly(vinylpyridine) polymer [19], ferrocyanide(II) [20], $[Ru(NH_3)_5\ py]^{2+}$ [21], o-phenylenediamine [22], tetrathiafulvalene [23], nickelocene [24] and tetracyanoquinodimethane salts [25] and quinone [26]. A typical trace of the steady-state current-time response of the sensor at an applied potential of - 0.20 V (vs SCE) shows that a subsequent addition of hydrogen peroxide to the solution provokes a sharp increase in the reduction current and that the response time is satisfactory, with the 95% steady-state response being achieved in less than 30 s, indicating that the sensor exhibits excellent bioelectrocatalytic activity to reduce hydrogen peroxide. Rapid response of the sensor to H_2O_2 is due to the highly effective ability of Meldola Blue to mediate the electron transfer between HRP and the carbon paste electrode. An extremely low detection limit of 1.0×10^{-7} moll can be estimated at a signal-to-noise ratio of 3.

3.2 Effect of the amount of Meldola Blue on the biosensor

Figure 1 shows the calibration curves for the biosensor. With the same amount of HRP, the concentration of Meldola Blue is the decisive factor in determining the sensitivity of the biosensor. Although direct electron transfer between HRP and the fumed silica-modified carbon paste electrode without mediator is observed, a comparison of the Meldola Blue-modified carbon paste biosensor for H_2O_2 with one without mediator based on the same amount of HRP

and fumed silica in the carbon paste, indicates that the Meldola Blue modified biosensor produces an approximately 5- to 15-fold higher reduction current than one without mediator. These facts indicate that in general the direct electron transfer between HRP and common electrode materials is a slow process and that the mediated electron transfer is more efficient in the bioelectrocatalytic reduction of hydrogen peroxide at the HRP-modified electrode. The dependence of the Michaelis-Menten constant K_m^{app} on the amount of Meldola Blue is summarized in Table 1. The enhancement of the sensitivity and Michaelis-Menten constant K_m^{app} with the amount of Meldola Blue is due to reinforced mediating ability.

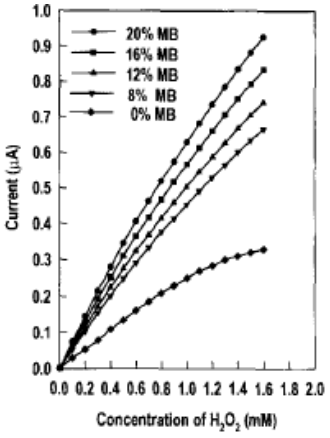

Fig. 1 Effect of the amount of Meldola Blue (MB) on the biosensor

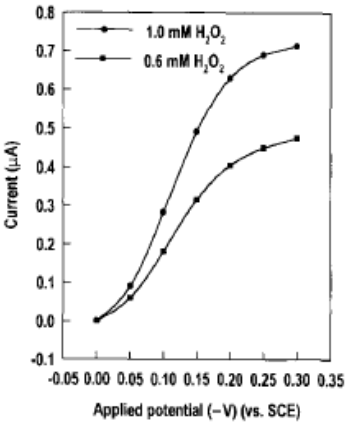

Fig. 2 Dependence of the biosensor current on the applied potential

Table 1 Effect of the amount of Meldola Blue (MB) on the Michaelis-Menten constant of the sensor at a applied potential of −200 mV (vs. SCE)

Amount of MB	8%	12%	16%	20%	24%
K_M^{app} (mmol/l)	1.32	1.45	1.68	1.86	1.92

3.3 Dependence of the biosensor on the applied potential

Figure 2 depicts the dependence of the biosensor current on the applied potential, indicating that the latter greatly affects the sensitivity. The enhanced sensitivity and linear range of the sensor with decreasing applied potential results from an increased driving force for the fast reduction of compound I and II. The Michealis-Menten constant of the sensor depends on the applied potential and increases from - 200 to - 300 mV (vs. SCE). The Michaelis-Menten constants (K_m^{app}) of the biosensor composed of 16% Meldola Blue are 1.68, 1.82 and 1.98 at applied potentials of - 0.20, - 0.25 and - 0.30 V, respectively.

3.4 Effect of pH and temperature on the sensor

The pH profile of the response of the sensor to hydrogen peroxide is shown in Fig. 3. An optimal pH of 6.5 was found, which reflected both the enzymatic and mediated electrochemical reactions in the carbon paste.

The effect of temperature on the sensor has been investigated between 10 and 55° C. The sensitivity increases with temperature, reaching a maximum value at 45° C. Further increasing temperature results in a decrease of the response current because of partial denaturation of the enzyme.

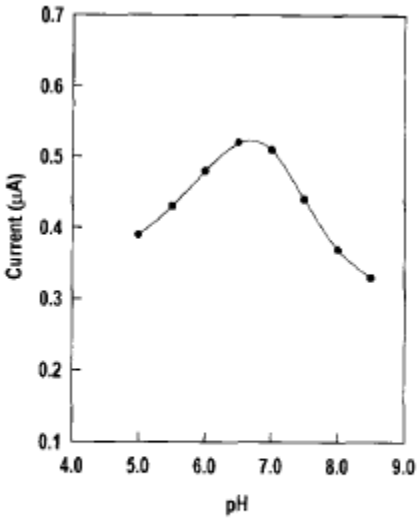

Fig. 3 Dependence of the biosensor current on pH. The experiment is conducted in 0.1 mmol/l phosphate buffer containing 0.8 mmol/l H_2O_2

3.5 Interferences

Investigations were made into the susceptibility of the biosensors to a range of possibly electrochemically interfering compounds by comparing the response of these compounds with that of 0.5 mmol/l H_2O_2 in 0.1 mol/l phosphate buffer. L-Tyrosine (0.2 mmol/1), L-lactate (0.5 mmol/1), galactose (5.0 mmol/l), L-leucine (0.2 mmol/1), L-cystine (0.2 mmol/1), L-tryptophan (0.2 mmol/1), L-cysteine (0.2 mmol/1), L-aspartic acid (0.2 mmol/1), L-histidine (0.2 mmol/l), glucose (5.0 mmol/1), uric acid (0.2 mmol/1), ascorbic acid (0.01 mmol/1) and L-glutamic acid (0.2 mmol/1) do not cause any observable interference with the determination of H_2O_2. Because of the Eastman-AQ polymer coating, a highly negatively charged polymer, anionic electroactive species such as uric acid and ascorbate are prevented from reaching the surface of the biosensor due to the charge repulsion between these compounds and the coating, and the interference from these species is suppressed. However, without the Eastman-AQ polymer coating, addition of ascorbic acid results in a decrease of the bioelectrocatalytic reduction currents, which may result from the reduction of the oxidized form of Meldola Blue and compound I and II by ascorbic acid. In addition, uric acid, a ubiquitous electroactive biomolecule, does not display any response at this biosensor without Eastman-AQ polymer coating.

3.6 Stability of the sensor

The storage stability of the sensor stored dry at 4°C has been examined by periodically checking its relative activity. The activity is maintained by 92.5% for a month and 81.6% for two months. This satisfactory operational stability was observed (indicated by the reproducibility of 3.6% and 3.1% relative standard deviations) by recording over 25 successive assays of 0.2 and 0.5 mmol/1 H_2O_2, respectively. The improved operational stability is due to a prolonged retention of HRP and Meldola Blue at the fumed-silica particles because of their huge surface area.

4. Conclusion

We have demonstrated that incorporation of fumed silica, HRP and Meldola Blue into carbon paste matrices greatly enhances the stability due to a prolonged retention of Meldola Blue and HRP. The sensor displays high sensitivity to hydrogen peroxide because of its high efficiency of bioelectrocatalytic reduction of hydrogen peroxide via Meldola Blue, an electron transfer mediator. We have applied this configuration to the design and preparation of a bienzyme system combining HRP with many oxidases for glucose, cholesterol, amino acids, glutamate, L-lactate, choline and uric acid. In addition, we have found that a series of compounds, such as methylene blue, phenazine methosulphate, cresyl fast violet, methylene green, catechol violet, methylene violet, brilliant cresyl blue, toluidine blue and new methylene blue N, are also able to enhance the electron communication between immobilized HRP and the carbon paste electrode. These results will be reported elsewhere.

Acknowledgements

This work is supported by the Natural Science Foundation of Shanghai for Young Scientists, the National Natural Science Foundation of China and the Open Electroanalytical Chemistry Laboratory of Changchun Institute of Applied Chemistry, Chinese Academy of Sciences.

References

1. Bonakdar M, Vilchez JL, Mottola HA (1989) J Electroanal Chem 266:47-55
2. Forzani ES, Rivas GA, Solis VM (1995) J Electroanal Chem 382:33-40
3. Kulys J, Gorton L, Dominguez E, Emneus J, Jarskog H (1994) J Electroanal Chem 372:49-55
4. Kulys J, Wang L, Maksimoviene A (1993) Anal Chim Acta 274:53-58
5. Wollenberger U, Wang J, Ozsoz M, Gonzalez-Romero E, Scheller F (1991) Bioelectrochem Bioenerg 26:287-296
6. Persson B (1990) J Electroanal Chem 287:61-80
7. Persson B, Gorton L (1990) J Electroanal Chem 292:115-138
8. Nagy G, Kapui I, Gorton L (1995) Anal Chim Acta 305:65-73
9. Wang J, Liu J (1993) Anal Chim Acta 284:385-391
10. Sprules SD, Hart JP, Wring SA, Pittson R (1995) Anal Chim Acta 304:17-24
11. Kulys J, Hansen HE, Buch-Rasmussen T, Wang J, Ozsoz M (1994) Anal Chim Acta 288:193-196
12. Kulys J, Schuhmann W, Schmidt H-L (1992) Anal Lett 25(6):1011-1024
13. Hurdis EC, Romeyn H Jr (1954) Anal Chem 26:320
14. Kamin RA, Wilson GS (1980) Anal Chem 52:1198
15. Scott DL, Paddock RM, Bowden EF (1992) J Electroanal Chem 341:307-321

16. Guo L-H, Hill HAO (1991) Adv Inorg Chem 36:341-375
17. Kulys J, Schmidt RD (1990) Bioelectrochem Bioenerg 24:305-311
18. Mulchandani A, Wang C-L, Weetall HH (1995) Anal Chem 67:94100
19. Garguilo MG, Huynh N, Proctor A, Michael AC (1993) Anal Chem 65:523-528
20. Liu Y, Liu H, Qian J, Yu T, Deng J (1996) Electrochim Acta 41:77-82
21. Frew JE, Harmer MA, Hill HAO, Libor SI (1986) J Electroanal Chem 201:1-10
22. Wang J, Wu L-H, Angnes L (1991) Anal Chem 63:2993
23. Bifulco L, Cammaroto C, Newman JD, Turner APF (1994) Anal Lett 27(8):1443-1452
24. Liu H, Qian J, Liu Y, Yu T, Deng J (1995) Anal Proceed 32:475-477
25. Korell U, Spichiger UE (1994) Anal Chem 66:510
26. Sanchez PD, Blanco PT, Alvarez JMF, Smyth MR, O'Kennedy R (1990) Electroanalysis 2:303